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(54) Title: ANTI-C3BI RECEPTOR (CR3) ANTIBODIES AND THERAPEUTIC COMPOSITION

(57) Abstract

An antibody is provided for use in the inhibition of recruitment of myelomonocytic cells to inflammatory stimuli, i.e. for treatment or prophylaxis of diseases and diseases states, e.g. inflammatory, autoimmune and hypersensitivity diseases, which arise from recruitment of myelomonocytic cells to inflammatory stimuli. The antibody is an antibody which has specificity for the type 3 complement receptor (CR3), in particular the α subunit thereof and is typically an antibody which is capable of inhibiting the attachment of myelomonocytic cells to bacteriologic plastic.

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ANTI-C3BI RECEPTOR (CR3) ANTIBODIES
AND THERAPEUTIC COMPOSITION

Field of the Invention

This invention relates to the field of immunology. In particular the invention relates to antibodies to cell surface receptors of myelomonocytic cells and to the therapeutic use of these antibodies to inhibit recruitment of myelomonocytic cells in response to inflammatory stimuli.

Background to the Invention

Myelomonocytic cells include monocytes, neutrophils and macrophages and are types of white blood cell. These cells constitute major components of the cell-mediated immune response system, providing the principal means of defence against bacterial and fungal infections. In addition, however, myelomonocytic cells are implicated in the pathogenesis of tissue damage in certain noninfectious diseases, including in particular some inflammatory and autoimmune diseases. These cells circulate within the vascular system and are recruited to sites of inflammation egressing from the vascular system and migrating towards the site of the inflammatory stimulus.

The mechanisms by which myelomonocytic cells are recruited to inflammatory sites is not well understood. However, recent studies have implicated certain types of cell surface molecule, in particular the Leukocyte Functional Antigen (LFA) family of surface receptors, in the adhesion of myelomonocytic cells to endothelial cells and the recruitment of these cells to inflammatory sites, (Springer, T.A., and D.C. Anderson (1986), Biochemistry of Macrophages, Ciba Foundation Symposium 118 pages 102-106; and Mentzer, S.J., M.A.V. Crimmins, S.J. Burakoff and D.V. Faller (1987) J. Cell Physiol. 130: 410).

The LFA family of cell surface receptors is made up of three structurally related heterodimeric glycoproteins, each having a unique higher molecular weight α subunit noncovalently linked to a common β subunit, which is structurally identical for all three family members. These LFA cell surface receptors are LFA-1

(comprising the CD11a α subunit and the common CD18 β subunit), CR3 (also known as Mac-1 or Mo 1) (CD11_b/CD18) and p 150, 95 (CD11_c/CD18). Studies (Pohlman et al, J. Immunol. (1986), Vol. 136, No. 12, pages 4548-4553) of neutrophil adherence to cultured human umbilical vein endothelial cells have indicated that an antibody (60.3) to the common CD18 β subunit inhibits adherence to a much greater extent than another antibody (60.1) having specificity for the α subunit of Mac-1 (i.e. CR3 or Mol).

In a recent in vivo study Arfors and co-workers (Arfors, K-E., C. Lundberg, L. Lindbom, K. Lundberg, P.G. Beatty and J.M. Harlan (1987) Blood 69: 338-340) have shown that a murine monoclonal antibody (60.3) directed against the CD18 complex, i.e. all members of the LFA family, inhibited both neutrophil accumulation and plasma leakage in rabbits. In this study relatively short-lived chemical stimuli were employed to bring about neutrophil accumulation and inflammation. The antibody used in these studies (60.3) appears to have specificity for an epitope present on the common β chain polypeptide of the CD w 18 complex, or an epitope formed by the quaternary structure of the α - β heterodimers (Wallis et al (1986) Blood 67: 1007).

Also in a further recent study (G. Ismail, M.L. Morganroth, R.F. Todd III and L.A. Boxer, Blood (1987), Vol. 69, No. 4, pages 1167-1174), it was shown, in an experimental model of neutrophil mediated lung injury, that preincubation of neutrophils with an anti Mol antibody prevented pulmonary injury by the activated human neutrophils in isolated perfused rat lungs. The monoclonal antibody used in this study, anti-Mol, had specificity for the α subunit of Mol (CR3/Mac1) and thus is specific for Mol (CR3/Mac1) not for the other members of the LFA family. The experimental model used in this study was based upon determination of endothelial cell damage at the vascular-endothelium surface interface not upon damage as the result of recruitment of neutrophils to extra-vascular sites of inflammatory stimuli.

We have now prepared monoclonal antibodies to thioglycollate-elicited peritoneal macrophages, have screened these antibodies in an in vitro assay and have identified a new sub-class of anti-CR3 specific antibodies which in vivo inhibit the recruitment of myelomonocytic cells in response to inflammatory stimuli.

The results we have obtained have given us insights into the mechanism by which myelomonocytic cells are recruited to sites of inflammation. Although both α and β subunits of the LFA receptors appear to be involved in adhesion of myelomonocytic cells to endothelium; it appears that CR3 alone plays a critical role in bringing about the egress of the myelomonocytic cells through the endothelial cell monolayer and subsequent migration to sites of inflammation.

A rat monoclonal antibody (M1/70), having specificity for an epitope on the α chain polypeptide of the type 3 complement receptor (CR3) component of the LFA family has been described (Springer, T., G. Galfre, D.S. Secher and C. Milstein, Eur. J Immunol. (1979) 9:301). However, in our hands this antibody has not proven to be useful for inhibiting in vivo recruitment of myelomonocytic cells to inflammatory stimuli, nor does it pass the in vitro screening assay which we have used to identify the new sub-class of anti-CR3 antibodies. Similarly we have tested an anti-Mol antibody (anti-Mol monoclonal antibody 44) as studied by Ismail et al (ibid) and found that this monoclonal antibody does not pass the in vitro screening assay.

Summary of the Invention

Accordingly, in a first aspect the invention provides an anti CR3 specific antibody for use in the inhibition of recruitment of myelomonocytic cells to inflammatory stimuli.

In a preferred embodiment the antibodies of the invention are capable of inhibiting the attachment of myelomonocytic cells to bacteriologic plastic in vitro in a screening assay as hereinafter described. Surprisingly we have found that there is a very strong correlation between the inhibition of attachment of myelomonocytic

cells to bacteriologic plastic which antibodies exhibit in the in vitro screening assay and the inhibition of recruitment of myelomonocytic cells to inflammatory stimuli which the antibodies are capable of causing in vivo. As indicated above, previously described anti-CR3 specific antibodies do not inhibit the attachment of myelomonocytic cells to bacteriologic plastic in the in vitro screening assay and thus this assay defines a new sub-class of anti-CR3 specific antibodies.

Accordingly, in a second aspect the invention provides an anti-CR3 specific antibody characterised in that the antibody inhibits the attachment of myelomonocytic cells to bacteriologic plastic in an in vitro screening assay as hereinafter described.

The in vitro screening assay which is used to identify and define the antibodies of the invention may be substantially as hereinafter described in detail. However, important features of the screening assay are as follows:

The cells used in the screening assay are characteristically myelomonocytic cells which have the CR3 receptor on their surfaces. For example, the cells used are macrophages and conveniently thioglycollate-elicited peritoneal macrophages (TPM) may be used.

The surface used in the in vitro screening assay is typically bacteriologic plastic or any other surface which has similar adhesion properties for CR3 bearing myelomonocytic cells. For example, the surface is an unmodified polystyrene plastic surface. Conveniently, unmodified polystyrene bacteriologic plastic micro-titre plates may be used in the screening assay. Tissue-culture treated plastic surfaces are generally not suitable for use in the screening assay.

The screening method used may involve incubation of cells with the bacteriologic plastic surface followed by fixation and then staining of the cells. The extent of adhesion may then be quantified by

recovery of stain from the cells and measurement by spectrophotometry. The inhibition of adhesion may be determined by comparison of the results obtained for untreated cells and cells which have been pretreated with antibody prior to adhesion.

Pretreatment with antibody may be carried out conveniently at a relatively low temperature, for instance, in the bacteriologic plastic micro-titre plate wells, e.g. 4°C for 30 minutes. However, the adhesion normally requires incubation at relatively higher temperature e.g. 37°C for 30 minutes. We have found that magnesium (Mg^{2+}), typically at a concentration of at least 100µM, is required during incubation with the bacteriologic plastic for satisfactory adhesion to take place. Conveniently the incubation medium may comprise Iscove's modification of Dulbecco's Medium containing 20% (v/v) foetal calf serum (FCS) or other suitable equivalent medium.

Characteristically the antibodies of the second aspect of the invention inhibit adhesion of myelomonocytic cells to bacteriologic plastic to the extent of at least 85%, preferably at least 90% especially at least 95%, and most preferably substantially completely when tested in an in vitro screening assay substantially as herein described.

In preferred embodiments the antibodies of the second aspect of the invention are capable of causing detachment of myelomonocytic cells from bacteriologic plastic, for instance, in an in vitro assay as hereinafter described. For example, the cells are attached to the bacteriologic plastic by suitable incubation e.g. 60 minutes incubation at 37°C, and are then incubated with medium containing a saturating concentration of the antibody for a suitable period of time, e.g. 5µg/ml for 30 minutes at 37°C.

The extent of detachment may be determined using a fixing, staining and spectrophotometric measurement method as for the adhesion assay. Preferred antibodies are typically capable of detaching at least 50%, especially about 65%, of thioglycollate-elicited

peritoneal macrophages or at least 75%, especially at least 85% of polymorphonuclear leukocytes in an in vitro detachment assay as herein specifically described, e.g. after exposure to a saturating concentration (5µg/ml) of antibody for a period of 30 minutes at 37°C.

Characteristically, the antibodies of the invention are capable of inhibiting the in vivo recruitment of myelomonocytic cells to inflammatory stimuli. The inhibition of in vivo recruitment of myelomonocytic cells may be determined by an in vivo assay as hereinafter described. Preferably the antibodies are capable of inhibiting in vivo recruitment of myelomonocytic cells to an extent similar to the specific antibody 5C6 as hereinafter described.

Particularly preferred antibodies are those having characteristics similar to or the same as the specific antibody 5C6 as hereinafter described. Thus characteristically the antibody has binding specificity for an antigenic epitope which is specific for CR3; for example an epitope of the α subunit of CR3. The epitope may be one requiring the presence of a β -chain, though is characteristically not one on the common β -chain polypeptide of the LFA family. The antibody is characteristically a CR3 specific antibody not an LFA family specific antibody.

Also, although the antibodies may be of any suitable immunoglobulin class or subclass, preferably they may be IgG, e.g. IgG2, or IgG2b antibodies. Preferably, also the antibodies are of relatively high affinity; for instance of affinity sufficient to inhibit attachment of myelomonocytic cells to bacteriologic plastic surfaces, e.g. as herein specifically described.

The antibodies of the invention may comprise monospecific antisera, or more preferably monoclonal antibodies or recombinant antibodies i.e. antibodies produced by recombinant DNA techniques, including chimeric, humanised, and CDR-grafted antibodies. Methods for the preparation of recombinant antibodies, chimeric antibodies, humanised antibodies and CDR-grafted antibodies are described in

published International Patent Applications Nos. WO 84/03712, WO 86/01533 and published European Patent Application No. EP 0239400.

The antibodies preferably comprise complete or substantially complete antibody molecules; though may comprise antibody fragments e.g. $F(Ab')_2$ fragments.

Advantageously the antibodies are obtained as monoclonal antibodies by cell fusion or other antibody-producing cell line immortalisation techniques. The antigen used to raise the antibodies characteristically comprises CR3. Myelomonocytic cells, e.g. thioglycollate-elicited peritoneal macrophages, may be used as the antigen, though purified and/or synthetic CR3 antigens e.g. purified CR3 α subunit glycoprotein or polypeptide or fragments thereof, may be used. The techniques for preparation of monoclonal antibodies are well known and understood by workers skilled in the art. Recombinant antibodies may be obtained by suitable manipulation of immunoglobulin genes and preparation of transformed host cells, also by techniques well known in the art. Genes for recombinant DNA manipulation may be obtained conveniently from suitable monoclonal antibody producing cell lines. Both monoclonal antibody and recombinant DNA techniques provide for establishment of cells which may be used to produce the antibodies of the invention.

In a third aspect the invention also includes a method for the preparation of a cell line which produces the antibodies of the second aspect of the invention comprising preparing a plurality of different cells which produce CR3 specific antibodies, screening the different cells for antibodies which inhibit adhesion of myelomonocytic cells to bacteriologic plastic and selecting cells which produce antibodies which inhibit adhesion.

Furthermore, in a fourth aspect the invention includes cell lines which produce antibodies according to the second aspect of the invention.

The antibodies of the invention may be used for therapeutic treatment of humans or animals. To be useful for treatment the antibodies used are typically not capable of causing aggregation, lysis or clearance of myelomonocytic cells, e.g. they do not fix complement or lyse cells. Also for treatment of humans the antibodies are preferably human, or humanised antibodies.

The antibodies may be used for treatment or prophylaxis of diseases or disease states which arise as the result of recruitment of myelomonocytic cells to inflammatory stimuli or as the result of complications of the adhesion of myelomonocytic cells to endothelium, such as increased vascular permeability. Diseases in which the recruitment of myelomonocytic cells appear to be involved in their development and or pathogenesis include inflammatory, acute hypersensitivity and autoimmune diseases. In particular the antibodies of the invention may be used in the treatment and prophylaxis of diseases involving recruitment of myelomonocytic cells in delayed type hypersensitivity reactions mediated by T-lymphocytes such as chronic inflammation and drug induced hypersensitivity reactions. Further examples of such diseases include rheumatoid arthritis, immune vasculitis, glomerulonephritis, and inflammatory bowel disease. Examples of other diseases which may be treated using the antibodies of the invention are: endotoxin toxicity, gout, immune complex diseases, multiple sclerosis and other inflammatory demyelinating diseases, neutrophil dermatoses, the after effects of myocardial infarction, adult respiratory distress syndrome, disseminated intravascular coagulation syndrome, emphysema, asthma, and the Arthus phenomenon. The antibodies of the invention may be used in treatment and prophylaxis in relation to these and similar diseases. It will be appreciated, however, that it may not be desirable to use the antibodies of the invention in disease states caused by rapidly proliferating acute infectious agents such as some types of bacteria.

Accordingly in a fifth aspect the invention provides a therapeutic composition comprising an antibody according to the first aspect of the invention in conjunction with a suitable excipient, diluent or carrier.

Therapeutic compositions for use according to the present invention may be formulated in conventional manner, optionally with one or more physiologically acceptable carriers diluents or excipients. The antibody compositions may be formulated for oral, buccal, parenteral or rectal administration or in a form suitable for nasal administration or administration by inhalation or insufflation.

Most usually, however, the antibody compositions are formulated for parenteral administration by injection e.g. by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use. Such parenteral compositions include compositions in the form of depot compositions which are long lasting.

More generally, depot compositions may be administered by implantation or by intramuscular injection.

In a sixth aspect the invention provides a process for the preparation of a therapeutic composition comprising admixing an antibody according to the first or second aspect of the invention with a suitable excipient, diluent or carrier.

Typically, the excipient, diluent or carrier is a physiologically acceptable excipient, diluent or carrier.

Furthermore, in a seventh aspect, the invention includes the use of an anti-CR3 specific antibody in the preparation of a medicament for inhibiting the recruitment of myelomonocytic cells to inflammatory stimuli.

Furthermore in a eighth aspect the invention provides a therapeutic method of inhibiting recruitment of myelomonocytic cells to

inflammatory stimuli in a human or animal subject by administering to the subject an effective amount of an antibody of the first aspect of the invention.

The amount of antibody which is administered to the human or animal subject will depend upon the properties of the antibody and the type of subject. Thus we have found in the case of the 5C6 antibody, as hereinafter specifically described, that a dose of about 0.5mg is sufficient to inhibit recruitment of myelomonocytic to inflammatory stimuli in a mouse for a period of up to 4 days. On this basis the dose required for a human subject is likely to be about 1g, typically in the range from 0.1 up to about 10g.

In accordance with the present invention, we have found that anti-CR3 specific antibodies may be used to inhibit the recruitment of myelomonocytic cells to inflammatory stimuli in vivo. We have identified that CR3 alone is critical for egress of myelomonocytic cells through the vascular endothelial cell monolayer and their subsequent migration to sites of inflammation. The use of anti-CR3 specific antibodies is particularly advantageous in comparison with use of antibodies to other members of the LFA family, such as antibodies to the common β subunit of LFA. CR3 is a surface antigen which is specific to myelomonocytic cells whereas other members of the LFA family are present on the surfaces of other types of cells of the immune system, including B lymphocytes and T lymphocytes. Thus use of an anti-CR3 specific antibody, as in our invention, specifically targets myelomonocytic cells and does not significantly effect other cells of the immune system which may lead to undesirable, generally comprising effects on the overall immune system. Advantageously, use of an anti-CR3 specific antibody leaves the remainder of the immune system substantially intact and functioning and capable of warding-off infectious and other invasive stimuli.

Brief Description of the Diagrams

The invention is further described by way of illustration only in the following Examples which refer to the accompanying diagrams, Figures 1 - 6, in which:

Figure 1 is a graph showing the results of quantitative adhesion assays after preincubation of cells in medium alone (C), 5C6 or M1/70 mAb (10^5 Thioglycollate elicited peritoneal macrophages (TPM) or bone marrow PMN (BM) were plated per well in 96 well plates on Bacterial (BP) or Tissue culture plastic (TCP). Results show the mean \pm SD of quadruplicates in three separate experiments after incubation for 30 min at 37°C , washing and Giemsa staining. Cell number was calculated from an internal standard of 10^5 adherent cells/well);

Figure 2 is a graph showing the results of quantitative assays of detachment by mAb 5C6 of TPM adherent to BP, or of bone marrow PMN (BM) adherent to TCP (Assay as described in Materials and Methods. Medium alone (C) or M1/70 supernatant (M1/70) failed to detach either TPM from BP or BM from TCP (results expressed as mean \pm SD of triplicates in two separate experiments));

Figure 3 is an autoradiograph of a 10% SDS-PAGE gel of ^{35}S -met labelled immunoprecipitate of cultured TPM (control preparation was precipitated with the rat anti-guinea pig PMN mAb 1A10.4 whilst both M1/70 and 5C6 precipitated a heterodimer of 165 and 95kDa that comigrated);

Figure 4 is a graph showing the effect of i.v. administration of 5C6 on the time course of increased footpad thickness in sensitised mice challenged with SRBC;

Figure 5 is a graph showing the time course of footpad swelling seen after challenge of actively immunised mice in the presence or absence of 5C6, and

Figure 6 is a graph showing the effects of 5C6 on the inflammatory recruitment of myelomonocytic cells following transfer of sensitised lymphocytes and tuberculin to the peritoneal cavity of naive, syngeneic mice.

Detailed Description of Embodiments of the Invention

The following Examples 1 and 2 refer to literature references by numbers in parentheses. These references are listed at the end of the Examples.

Example 1

This Example describes the preparation, in vitro screening, characterisation and in vivo testing of an antibody according to the invention.

Materials and Methods:Cells

Murine thioglycollate peritoneal macrophages (TPM) and polymorphonuclear leucocytes (PMN) were harvested from the peritoneal cavity by lavage 4 and 1 days respectively after the intraperitoneal (ip) injection of 1ml Brewer's complete thioglycollate broth. Cytospin preparations stained with Giemsa revealed that ~ 80% of the 4 day exudate (3×10^7 cells) were PMN. Bone marrow cells, ~ 45% PMN, were obtained by flushing the femoral cavity with phosphate-buffered saline (PBS). BCG activated and resident peritoneal M ϕ and Kupffer cells were isolated as described (1).

Animals

Mice (C57/B1 or Pathology Oxford, PO), of either sex, were bred at the Sir William Dunn School of Pathology. AO rats (specific pathogen free) were from the MRC Cellular Immunology Unit in our department.

Adhesion assays

Cells were suspended in Isove's modification of Dulbecco's Medium with 20% fetal calf serum (FCS) and plated at a density of 10^5 cells/well in 96 well plates that were either flat-bottomed bacteriologic plastic (BP) (Flow Laboratories, Rickmansworth, Hertfordshire) or tissue-culture treated plastic (TCP) (Sterilin, Ashford, Middlesex). After incubation for 30 min at 37°C, plates

were washed three times in PBS and adherent cells fixed with methanol. After staining with 10% Giemsa solution for 10 min, plates were washed in tap water, dried and the retained dye solubilised in methanol. Stain was quantified by measuring absorbance at 460nm in an automatic plate reader (Dynatech, Alexandria, Va.). This assay was linear between 5×10^3 and 10^5 adherent cells per well. In antibody (ab) inhibition experiments, hybridoma conditioned media were added to microtitre plates and cooled to 4°C before addition of cells in the same medium. After preincubation at 4°C for 30 min, neither ab treated nor control cells had adhered and the plates were then warmed to 37°C for 30 min before washing and processing. In detachment assays 10^5 Mφ were plated in serum containing medium and allowed to adhere for 60 min at 37°C . After washing to remove non-adherent cells, ab was added in medium and cells incubated at 37°C for 30 min. Thereafter, plates were washed and processed as above and the percentage of detached cells calculated. The effects of various inhibitors on adhesion were studied by preincubating cells in one or more of 5μM cytochalasin B (CB), 10μM/ml colcemid or 5mM 2-deoxyglucose for 30 min at 4°C before performing the adhesion assay in medium with 10% FCS as described above. Experiments assessing the cation requirements of adhesion were carried out in Iscove's medium and 10% FCS with either 5mM Ethylenediaminetetracetic acid (EDTA) or 5mM Ethylglycolbisaminoethylethertetracetic acid (EGTA) to restrict Ca^{2+} and Mg^{2+} or Ca^{2+} alone. The absolute cation requirements were determined by performing adhesion assays in Ca^{2+} and Mg^{2+} free Hanks balanced salt solution (HBSS), with 10% FCS dialysed to remove divalent cations and defined quantities of added CaCl_2 or MgCl_2 . The effect of surface proteolysis on adhesion was assessed by treating cells in suspension with 0.5mg/ml Pronase (Boehringer, Lewes, Sussex) or 0.1mg/ml crystalline trypsin in Iscove's medium without added protein for 30 min at 37°C . After washing the cells in 10% FCS, they were plated in serum-containing medium on the appropriate substratum and assayed in the standard adhesion assay.

Antibody production

mAb 5C6 was the product of a fusion between spleen cells from an AO rat, immunised four times with TPM, and the Y3 rat myeloma line. The fusion was performed as described by Galfre et al (2). Hybridoma supernatants were screened for inhibition of attachment and for detachment of TPM. One positive culture out of 1056 wells was isolated and cloned twice by limiting dilution. Antibody was purified from ascites by sodium sulphate precipitation, anion exchange chromatography on DEAE-Sephacel (Pharmacia, Hounslow, Middlesex) and gel filtration chromatography on S-300 Sephadex (Pharmacia). Purity was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels using the buffer system of Wyckoff et al (3) and staining with Coomassie Blue. The IgG was free of any other protein bands when run either reduced or non-reduced. F(ab')₂ fragments were prepared by pepsin digestion of IgG at pH 4.0 in 0.1M acetate after Rousseaux et al (4) and purified by gel filtration chromatography. Fab fragments were produced by papain digestion of IgG in 75mM Na phosphate pH 7.0/75mM NaCl and purified by anion exchange chromatography on DEAE-sephacel. 5C6 is a rat IgG2b as typed by Ouchterlony gel diffusion using antisera directed against rat IgG subclasses supplied by Dr. H. Bazin (Catholic University, Louvain). 5C6 was fluoresceinated by the method of Mason (5).

Labelling, immunoprecipitation and SDS-PAGE

4 day TPM were plated at 10^7 cells/10cm culture dish and cultivated overnight in methionine-free medium with 200 μ Ci-³⁵S-methione. After a chase incubation with unlabelled methionine, the cells were washed in PBS and lysed in 1% v/v Triton X-100 in PBS, 10mM EDTA, 3mM Phenyl methyl sulfonyl fluoride (PmsF) and 3mM iodoacetamide. After preclearing the lysate with protein A-sepharose, mAb in detergent was added for 60 min at 4°C. Thereafter the mAb-ag complex was precipitated with rabbit IgG anti-rat IgG-Protein A-sepharose. The beads were washed by standard methods and boiled in sample buffer containing 2% SDS. The eluates were analysed by electrophoresis on 10% polyacrylamide gels and autoradiography. Control preparations contained a mAb to

determinants not found on mouse Mφ. In peptide mapping experiments, 5C6 and M1/70 immunoprecipitates on protein-A-sepharose beads were digested for varying times at 37°C with 100µg/ml crystalline trypsin. The reaction was stopped by boiling the beads in SDS sample buffer and the resultant cleavage patterns analysed by SDS-PAGE.

Preparation of derivatised substrate

Fibronectin-coated surfaces were prepared by coating tissue-culture plastic surfaces with gelatin followed by fresh mouse serum for an hour at room temperature. The wells were then extensively washed with PBS. Poly-L-lysine coated, glutaraldehyde activated surfaces were derivatised with Dinitrophenyl (DNP) Bovine serum albumin (BSA)-anti-DNP immune complexes as described (6).

Chemotaxis Assay

The directional migration of myelomonocytic cells in response to the chemotactic tripeptide formyl Methionyl Leucyl Phenylalanine (fMLP) was assayed under agarose as described (7).

Recruitment Assays

PO mice were injected intravenously (iv) with PBS, 5C6 IgG F(ab')₂ or the anti-L3T4 subclass matched (IgG2b) YTA mAb, 4 hours prior to injection of 1ml thioglycollate broth ip. All ab injections were 0.5mg unless stated otherwise. At 18 or 96 hours after first injection, mice were killed and peritoneal exudate cells, blood, livers, spleens and bone marrows removed. Total and differential counts of exudate cells, bone marrow cells and blood leukocytes (after sedimentation of erythrocytes with 1% Dextran T500) were obtained using May-Grunwald-Giemsa stain. Inhibition of recruitment by mAb was calculated as total exudate cells - resident cells in Ab-treated mice divided by recruited cells from control mice. Cells were then tested for adhesion to BP or TCP. Livers and spleens were examined histologically as follows. In some experiments tissues were fixed in glutaraldehyde and stained with haematoxylin and eosin. In experiments where fluorescein isothiocyanate (FITC)-5C6 had been injected in vivo, organs were

examined for the presence of mAb by direct immunofluorescence of frozen sections (5µm) embedded in Tissuetek (Miles Laboratories, Naperville, Il). Alternatively, animals were perfusion fixed as described (8) and frozen sections of tissues analysed by the immunoperoxidase technique without adding exogenous first ab to the sections.

Rosetting assays

Sheep erythrocytes (E) were opsonised with IgM ab and iC3b or C142 (9), or rabbit-anti-sheep E IgG and used as a 5% v/v suspension for rosetting at 4°C or phagocytosis at 37°C by adherent TPM (10). Binding was quantified by counting the number of attached erythrocytes to 100 TPM by phase contrast microscopy following washing and glutaraldehyde fixation.

Microscopy

Fluorescence microscopy was performed using a Zeiss Axiphot epifluorescence microscope. Scanning electron microscopy was performed as described (10).

Reagents

M1/70 (Springer, T. et al *ibid*) a rat anti-CR3 mAb was used either as IgG or hybridoma supernatant from laboratory stocks. The cell line was the gift of Dr. T. Springer (Dept. Pathology, Harvard Medical School, Boston). YTA mAb was a gift of Dr. H. Waldmann (Dept. Immunology, University of Cambridge). Rabbit-anti-E IgM (EA) and EA iC3b were provided by Dr. R. Sim (MRC Immunochemistry, University of Oxford). Reagents were obtained from the following sources: Iscove's medium (Flow); FCS (Gibco, Paisley, Scotland); Colcemid (Dr. E.P. Evans, Sir William Dunn School of Pathology, Oxford); Deoxyglucose, cytochalasin B, EDTA and EGTA (Sigma, Poole, Dorset); Protein-A-Sepharose (Pharmacia).

Protein Assays

Protein concentrations of IgG were assayed according to Lowry *et al* (11) using BSA as standard.

RESULTS:Adhesion Assays

We characterised M ϕ and PMN adhesion in a variety of simple, reproducible short term adhesion assays to develop a screening method for mAb directed against functional epitopes of phagocyte surface molecules. Adherence of various primary murine M ϕ to tissue-culture treated plastic (TCP) or bacteriologic plastic (BP) (polystyrene) was examined because selective adherence of M ϕ to these substrata forms the basis of their rapid purification from other blood cells and fibroblasts (12). All assays were carried out in the presence of serum. The comparative adhesive qualities of different cells to the plastic substrata are shown in Table I. The adhesion of M ϕ and exudate PMN to BP was Mg²⁺-dependent, pronase sensitive and required elevated temperature as well as cytoskeletal stabilisation for efficient binding. Exposure of TPM to 0.5mg/ml pronase at 37°C for 30 min completely abolished their ability to adhere to BP. This effect persisted for at least 6 hours and cells recovered fully by 24 hours in culture. Resting PMN and tissue M ϕ such as Kupffer cells were unable to adhere to BP. Adhesion to BP required at least 100 μ M extracellular Mg²⁺ and Ca²⁺ alone was unable to substitute for this requirement. The adhesion of PMN (whether resting or exudate) to TCP had the same qualities as M ϕ adhesion to BP. All cell types tested adhered well to TCP and showed varying degrees of spreading. As in BP adhesion, M ϕ adhesion to TCP required elevated temperature and an intact cytoskeleton, but differed in being resistant to proteolysis and was partially cation-independent.

TABLE I

Adhesion of cells in vitro after 30 min at 37°C in 10% FBS.
Absorbance of Giemsa at 460nm was used to determine adherent cell numbers.

<u>Cell type and Treatment</u>	<u>Tissue Culture Plastic</u>		<u>Bacterial Plastic</u>	
	<u>Absorbance</u>	<u>Cell</u> <u>number</u> 10^{-3}	<u>Absorbance</u>	<u>Cell</u> <u>number</u> 10^{-3}
Resident Peritoneal Mφ	0.174	96±4	0.135	74±3
Thioglycollate PMφ	0.189	104±5	0.147	81±5
Kupffer Cells	0.181	99±3	0.002	1±3
Bone marrow PMN	0.180	99±3	0.008	4±3
Exudate PMN	0.186	102±4	0.151	83±5
<u>Cation requirement (TPM)</u>				
Mg ²⁺ present				
(No Ca ²⁺)	0.161	88±4	0.118	65±3
Ca ²⁺ present				
(No Mg ²⁺)	0.104	57±5	0.004	2±2
Neither present	0.104	57±5	0.004	2±2
<u>Protease sensitivity (TPM)</u>				
Trypsin	0.178	98±3	0.152	84±4
Pronase	0.182	100±2	0.005	3±2
<u>Metabolic and Cytoskeletal Factors (TPM)</u>				
Controls (37°C, no inhibitors)	0.152	84±4	0.135	74±3
Temperature < 40°C	0.008	4±3	0.004	2±2
Cytochalasin B 5μM	0.072	40±3	0.058	32±3
Colcemid 10μg/ml	0.063	35±5	0.049	27±4
Cytochalasin B + Colcemid	0.006	3±2	0.002	1±2
2-Deoxyglucose	0.151	83±3	0.051	28±1

Results of quadruplicates in three separate experiments are expressed as the mean absorbance and the mean ± SD of adherent cell number calculated from an internal standard. ~ 1x10⁵ myelomonocytic cells were plated/well.

The adherence of M ϕ to BP appeared to be a useful means of identifying a pronase-sensitive, cation-dependent surface component involved in cell adhesion. We thus used this assay to screen for functional mAb capable of, first, inhibiting attachment of TPM to BP and, second, detaching adherent TPM from BP.

mAb 5C6 inhibits adhesion to BP and detaches adherent TPM

In each of two consecutive fusions, one hybridoma secreting a mAb capable of inhibiting attachment of TPM to BP was isolated. Both identify the same antigen and this report is confined to the first of these mAb designated 5C6. The effects of mAb 5C6 on adhesion of TPM to bacterial and tissue-culture treated plastic was investigated. We found that untreated 4 day TPM adhered and spread on BP after 30 minutes at 37°C in serum-containing medium. Preincubation of these cells in 5C6 hybridoma supernatant for 30 minutes at 4°C before warming to 37°C to allow adhesion, led to the complete abolition of TPM attachment to BP. Whilst untreated TPM adhered and spread on TCP after 30 min at 37°C cells preincubated with 5C6 showed unimpaired adhesion to TCP, but did not spread at all.

The adhesion of untreated TPM to glass was similar to that observed on TCP. Control TPM adherent to glass was flat and well spread. After these cells had been preincubated in 5C6 mAb, the cells adhered via a relatively small, snail-like foot process whilst the bulk of the plasma membrane remained in a highly ruffled dome over the cell body. Adhesion of M ϕ to glass and TCP had two phases, mAb 5C6-resistant adherence followed by mAb 5C6-sensitive spreading.

TABLE II

A comparison of the functional characteristics of 5C6 Mab (intact IgG, F(ab')² and Fab fragments) and M1/70 IgG.

	5C6 IgG	5C6 F(ab') ²	5C6 Fab	M1/70 IgG
Inhibition of TPM adhesion to BP	92 ± 1%	51 ± 3%	0%	- 22%
Detachment of adherent TPM (BP)	65 ± 4%	8 ± 3%	5 ± 2%	6 ± 3%
Inhibition of E1C3b rosetting	> 95%	> 95%	0%	> 95%
Pronase	85 ± 1%	ND	ND	26 ± 1%

5C6 IgG, F(ab')² and Fab were used at 1, 10 and 100µg/ml. Rosetting was quantified as described in Methods. Results reflect the mean ± SD of at least 3 separate experiments.

TPM were digested in suspension (30 min, 37°C) with 0.5mg/ml pronase, fixed in 0.25% glutaraldehyde, quenched in FCS and 1 x 10⁶ cells assayed in quadruplicate by trace indirect binding assay (2 experiments). Untreated cells bound 3903 ± 47 cpm of second ab after binding of 5C6 and 3336 ± 51 cpm after pronase digestion. Untreated cells bound 3292 ± 35 cpm using M1/70 as first ab and only 866 ± 12 cpm after pronase treatment. The Mφ-specific F4/80 ag known to be pronase-sensitive was abolished by this treatment.

Fig. 1 and Table II summarise quantitative assays of adhesion in the presence or absence of mAb and show that 5C6 IgG at 0.5µg/ml inhibited attachment of TPM to BP by $92 \pm 1\%$ whilst untreated cells or cells treated with saturating concentrations of the anti-CR3 mAb M1/70, used for comparison, displayed unaltered adhesive capacities. The increased adherence of M1/70-treated cells as compared to untreated cells reflected aggregation of cells by this ab and adhesion of these clusters. In addition to its effects on Mφ adhesion, 5C6 also abolished the adhesion of PMN to TCP, which was unimpaired in control or M1/70-treated PMN. Whilst M1/70 treatment led to aggregation of myelomonocytic cells in suspension, 5C6-treated PMN or Mφ remained discrete.

The CSAT ag or Fibronectin receptor of avian fibroblasts was identified by the ability of ab to detach cells adherent to a fibronectin-coated substratum (13). Following this precedent, we examined the ability of 5C6 to detach TPM from BP or bone marrow PMN from TCP. After allowing cells to attach and adhere for 60 minutes at 37°C, plates were washed and medium containing saturating concentrations (5µg/ml) of 5C6 or M1/70 or no ab was added. Within 5 minutes at 37°C, spread cells treated with 5C6 had become rounded whilst M1/70-treated and control cells were unaltered. By 30 minutes at 37°C, (Fig. 2) 65% of 5C6-treated TPM and > 85% of 5C6-treated PMN were detached. This differed substantially from the 3% of M1/70-treated TPM or PMN detached in the same period. Detachment by lower concentrations of mAb (0.05µg/ml) was slower with 30 min needed for rounding of adherent cells and 90 min for detachment. The attachment of TPM to defined substrata such as fibronectin or immune complexes was unaffected by 5C6 mAb.

mAb 5C6 binds to CR3 and inhibits iC3b binding

The ag recognised by 5C6 was characterised by the cell binding profile of the mAb and the molecular species identified by immunoprecipitation. Indirect binding radioimmunoassays with fixed cells and direct immunofluorescence studies of live cells revealed that 5C6 bound to a methanol- and glutaraldehyde- stable epitope on TPM, RPM, BCG-activated Mφ and PMN, that was absent from the surface of Kupffer cells (as gauged by immunoperoxidase staining)

and from resting or BCG-activated lymphocytes.

Immunopurification of 5C6 ag from ³⁵S-met labelled TPM revealed a heterodimer of 165 kDa and 95kDa which comigrated with CR3 as precipitated by M1/70 (Fig. 3). Further proof of the identity of these molecules was that the 165 kDa chain precipitated by 5C6 peptide mapped identically to the α -chain of CR3 after limited tryptic cleavage. In addition, like intact M1/70, both 5C6 IgG and its F(ab')₂ fragment, but not Fab, inhibited rosetting of EAiC3b to TPM (Table II) whereas no inhibition of rosetting of EAC142 or EIgG (FcR) was seen. Maximal inhibition of EAiC3b rosetting was obtained with 0.35 μ g/ml of 5C6 IgG.

The 5C6 epitope is distinct from the M1/70 epitope

The disparity between 5C6 and M1/70 in their ability to inhibit adhesion of TPM to BP led us to explore the possibility that the two ab identify distinct epitopes on the CR3 molecule. We therefore compared the sensitivity of the binding of these ab to proteolytic digestion of the TPM cell surface. Digestion of the cell surface with pronase (0.5mg/ml for 30 min at 37°C) destroyed most of the M1/70 binding sites and abolished the M ϕ -specific F4/80 epitope whereas the 5C6 sites were more resistant to proteolysis (Table II).

The 5C6 antigen, like that of M1/70, is probably an α -chain epitope as it was restricted to myelomonocytic cells and was absent on lymphocytes, that are rich in LFA-1 which shares a common β chain with CR3. The 5C6-epitope might require α chain together with the common β chain since after dissociation of chains by pH 11.5 in solution, 5C6 failed to precipitate any ag. The 5C6-epitope was also dissociated from the adhesive domain of CR3 because pronase digestion abolished adhesion of TPM to BP whilst binding of 5CE was largely intact (cf Table I).

Optimal inhibition of adhesion requires intact IgG

We examined the ability of defined immunoglobulin fragments to alter M ϕ adhesion to BP in vitro. Intact 5C6 IgG inhibited adhesion of TPM by 92% compared with untreated cells (Table II). The F(ab')₂

fragment of 5C6 inhibited ~ 50% adhesion despite binding comparably to the intact IgG in indirect binding assays. The Fab fragment also bound well showing 50% saturation at 0.1µg/ml of protein, but did not inhibit TPM adhesion. Interestingly, in experiments using purified 5C6 IgG at concentrations of 0.05mg/ml where monovalent binding becomes significant, functional activity of the IgG diminished in a prozone-like phenomenon. It was clear that divalent binding was thus a prerequisite but alone was insufficient to inhibit adhesion. Only the intact 5C6 IgG was able to reverse adhesion but detachment under these conditions was partial.

The effect of mAb on recruitment of myelomonocytic cells to an inflammatory site in vivo

We next examined the possible role of CR3 in the recruitment of myelomonocytic cells to the peritoneal cavity. Intravenous injection of mice with purified 5C6 IgG was followed four hours later by an intraperitoneal challenge of sterile thioglycollate broth. A total of 22 5C6 IgG-injected mice and 20 control mice were examined in this series of experiments (Table III).

TABLE III

Injection of 5C6 IgG in vivo inhibited recruitment of myelomonocytic cells to a peritoneal exudate

- A. The effect of treatments on 18 hour peritoneal exudates and blood leukocytes. The treatment preceded thioglycollate injection IP by 4 hours in all cases.

<u>Pre Treatment</u>	A <u>Peritoneal</u> <u>Cells</u> 10^{-6}	B <u>Exudate</u> <u>Differential</u>	C <u>Blood</u> <u>Cells</u> 10^{-6} /ml	D <u>Differential</u>
PBS iv or nothing (16 mice)	36±4	54P 40M 6L	16±2	80P 12M 8L
0.5mg 5C6 IgG iv (18 mice)	9.0±2	35P 51M 14	17±2	77P 13M 10L
0.5mg 5C6 F(ab') ² iv (2 mice)	26	71P 29M	15	72P 13M 15L
0.5mg YTA IgG iv (2 mice)	22	65P 33M 2L	14	75P 10M 15L

- B. The effects of 56C IgG treatment on 4 day peritoneal exudates and blood leukocytes

<u>Treatment</u>	<u>Peritoneal</u> <u>Cells</u> 10^{-6}	<u>Exudate</u> <u>Differential</u>	<u>Blood</u> <u>Cells</u> 10^{-6} /ml	<u>Differential</u>
PBS iv (4 mice)	27±4	P25 M66 L9	9.1	55P 12M 33L
0.5mg 5C6 IgG	9.4±2	P8 M38 L54	8.6	52P 10M 38L

Controls

Animals not exposed to thioglycollate broth, with or without 5C6 IgG pretreatment, yielded $5-6 \times 10^6$ cells, 35% M 65% L. Blood and bone marrow cell counts were also unchanged from controls.

In control animals which received nothing or PBS alone, recruitment of cells to the peritoneal cavity 18 hours after thioglycollate injection was unimpaired. Cell yields were enhanced 3-4 fold over untreated controls and consisted predominantly of PMN and M ϕ . By 4 days the recruited peritoneal cell yields had dropped by 25% with a higher proportion of M ϕ and fewer PMN. In 2 mice injected intravenously with 0.5mg of YTA, an isotype matched Mab directed against CD4 antigen, 22×10^6 peritoneal cells were recovered at 18 hours compared with 26×10^6 cells recovered from PBS injected animals in the same experiment, showing a small diminution of recruitment. In striking contrast, peritoneal recruitment in the 5C6 IgG injected animals was reduced by $84 \pm 3\%$ at 18 hours, whereas peritoneal yields in animals which received the mAb, but no thioglycollatae broth, were unaffected. Inhibition of recruitment persisted for at least 4 days in 5C6 IgG-treated animals. From differential counts we calculated that 3.1×10^6 PMN were recovered at 18h in ab-treated mice compared with 2.0×10^7 in controls and 3.5×10^6 M ϕ after 4 days, compared with 1.8×10^7 in controls. The ability of 5C6 intact IgG to inhibit recruitment of myelomonocytic cells correlated with its inhibition of adhesion in vitro whereas the F(ab')₂ fragments which showed only partial inhibition of adhesion in vitro, failed to impair recruitment in vivo. The inhibition of recruitment by 5C6 IgG was dose dependent: 0.5mg IgG iv was the smallest dose of IgG able to inhibit peritoneal recruitment > 80% at 18 hrs and inhibition of recruitment by 0.1, 0.2 and 0.35mg was 40%, 51% and 68% respectively (One experiment, 2 mice per data point).

Peritoneal cells recovered after iv treatment with mAb adhered well to broth BP and TCP in vitro, suggesting that their exposure to 5C6 had been subsaturating. Experiments in which 5C6 mAb was injected directly ip, resulted in binding of ab to all peritoneal M ϕ which, when harvested, had lost their ability to adhere to BP or spread on TCP, like M ϕ treated with mAb in vitro.

The blood, bone marrow, liver and spleen of control and mAb-treated mice were examined to identify its site of action. There was no

difference in number or differential counts of femoral marrow cells, between control and 5C6-treated mice. Marrow plugs were lightly fixed in paraformaldehyde, embedded and stained by the immunoperoxidase method after frozen section to detect mAb which had bound in vivo. Marrows from 5C6-treated mice showed heavy labelling of most mature myelomonocytic cells. Blast cells, lymphocytes and resident bone marrow Mφs remained unlabelled, as expected. There was no evidence of cell death.

Columns C and D of Table III show the analysis of blood leukocytes in control and experimental animals. Thioglycollate broth elicited a PMN leukocytosis at 18 hours which was similar in ab-treated and untreated mice. In mice which had not been exposed to thioglycollate, with or without 5C6 IgG pretreatment, there were $7-10 \times 10^6$ cells/ml, 60-70% PMN. These data showed that the egress of myelomonocytic cells from marrow into blood had not been impaired. Furthermore, blood leukocytes in both control and 5C6 IgG-treated mice were not aggregated. Blood PMN from control animals adhered well to BP and TCP in vitro whereas blood leukocytes from 5C6-IgG-treated mice failed to adhere to either substratum. Histological analysis of sections of liver and spleen showed no evidence of leukocyte aggregation, cell death or removal by phagocytosis. Particular attention was paid to the examination of liver sinusoids and the splenic red pulp and no differences were discerned between control and mAb-treated animals. These observations made it unlikely that excessive PMN margination or phagocytic clearance was responsible for the failure to recruit myelomonocytic cells to the peritoneal cavity.

The observed inhibition of recruitment by ab could be due to inhibition of adhesion of circulating cells to endothelium and/or failure of marginated cells to undergo directional migration into the tissue spaces. We therefore examined the ability of bone marrow leukocytes from 5C6-treated and control mice to undergo chemotaxis in response to fMLP in an under-agarose assay (6 animals, 3 experiments). There was no difference in the distance migrated by bone marrow PMN from 5C6-IgG-treated or control mice (not shown) indicating that the mAb treatment in vivo had not impaired the ability of leukocytes to respond to a defined chemotactic stimulus.

EXAMPLE 2

This example describes investigations of the extent to which T-cell dependent inflammation induced by specific antigen (ag) challenge in sensitised mice, or by adoptive transfer in naive, syngeneic mice is inhibitable by a monoclonal antibody (5C6) according to the invention, directed specifically to CR3.

MATERIAL AND METHODSMice

8 week old C57/BL6 female mice were obtained either from the Sir William Dunn School of Pathology, Oxford, or the specific pathogen-free unit of the Institut Pasteur, Paris.

Immunisation and treatment of mice

Sheep erythrocytes (SRBC) in Alsevers solution (GIBCO, Paisley, Scotland) were washed three times in Dulbecco's A phosphate buffered saline (PBS) and resuspended in sterile normal saline. 10^5 SRBC were injected i.v. into the tail vein. Mice were immunised with 3×10^6 viable BCG organisms (Institut Pasteur) (14) by subcutaneous injection in both hind footpads.

Assay of the DTH to SRBC

Four days after immunisation mice were tested for DTH by an antigenic challenge of 10^7 or 10^8 SRBC injected subcutaneously in a volume of 50 μ l into the right hind footpad. Footpad swelling was measured at a range of times with a dial gauge caliper and the measurement of the uninjected footpad was subtracted from that of the challenged footpad. Footpads were subsequently removed and fixed in formol-saline. The wax-embedded footpads were then sectioned and stained with haematoxylin and eosin. The local adoptive transfer of the DTH reaction in syngeneic naive mice was performed as described (15). Briefly, four days after immunisation, mice received 50 IU of heparin. Heparinised blood was collected fifteen minutes later, diluted and used as a source of SRBC-sensitised T-cells. This was then mixed with either SRBC or

unrelated ag and injected into one hind footpad of naive, syngeneic recipients and the increase in footpad thickness examined 14-18 hrs later. Footpads were processed for histological examination as above.

Assay of the adoptively transferred inflammatory response to Tuberculin

Four to six days after BCG immunisation of the footpads, draining lymph nodes were removed, teased apart and the single cell suspension used as a source of tuberculin reactive T-lymphocytes. 1.5×10^7 cells were mixed with 50µg of tuberculin and injected in a volume of 1ml into the peritoneal cavity of naive, syngeneic mice (16). Peritoneal cells were harvested 48 hours later, counted and cytospin preparations were then examined immunocytochemically by an indirect immunoperoxidase technique (17) to allow an accurate assessment of the different cells recovered. Specifically, the proportion of macrophages (Mφ) was assessed by staining cytospin preparations for the F4/80 ag (18) with a polyclonal rabbit antiserum prepared against purified murine F4/80 ag. This permitted the specific staining of Mφ even when labelled with the 5C6 rat mAb.

The effects of mAb 5C6 pm expression of DTH

5C6, a rat IgG2b mAb directed against the murine CR3 was prepared and purified as described in Example 1. 1mg of IgG was injected i.v. either at the time of ag challenge or up to 6 hrs later and the effects compared with controls in which buffer alone, a control rat IgG2a mAb 7/4 (19) restricted to murine myelomonocytic cells and with a similar site number to 5C6 or the F(ab')₂ fragment of 5C6 that has no in vivo activity, was injected. In experiments where local injection of sensitised T-cells and ag was used to adoptively transfer DTH, 5C6 or a control preparation was injected i.v. to donor mice at the time of immunisation to assess possible effects on the development of ag-reactive T-cells. Recipients were injected with mAb i.v. at the time of local transfer to influence systemic delivery of inflammatory cells. Alternatively, the transferred blood cells were preincubated with mAb and then washed by centrifugation prior to local injection.

The persistence of mAb following a simple i.v. injection

The adequacy of circulating 5C6 IgG was assessed in two ways. First, serial blood samples were taken at times from 1 hr to 4 d following a single i.v. injection of 1 mg. The serum was then diluted in Iscove's medium (Flow Laboratories, Paisley, Scotland) with 5% fetal bovine serum and 4-day thioglycollate-elicited Mφ were then incubated in the diluted serum before measurement of adhesion to bacterial plastic as described in Example 1. 4 days after injection, a 1:10 dilution of the sampled serum still inhibited adhesion to plastic by > 85%. In addition, the degree of mAb binding to blood leukocytes, peritoneal cells and bone marrow cells was assessed at 1, 4, 24, 48 and 72 hrs after a single i.v. injection of FITC-5C6, by fluorescence analysis on a Becton-Dickinson FACS II apparatus. Saturation binding to myelomonocytic cells alone was seen, and persisted for the duration of the experiment.

Assay of the half-life of 5C6 IgG and F(ab')₂ in the circulation

Mice were injected i.v. with 3000 units of intact IgG or F(ab')₂. 50μl of blood were collected from the tail veins at 10 minutes, 2hrs, 4hrs, 8hrs and 24hrs after injection. Serial dilutions of this blood were assayed for binding activity as described in Example 1. Binding was defined in an indirect radio-immunoassay to adherent glutaraldehyde-fixed thioglycollate-elicited macrophages. The reciprocal of the dilution of antibody that gives rise to 50% maximal binding of a fixed quantity of iodinated second antibody is designated the number of binding units per ml. In this assay 1mg/ml of pure 5C6 IgG contains 6300 binding units.

RESULTS:Effect of mAb 5C6 on DTH following active immunisation with SRBC

There is convincing evidence that the footpad DTH measured in the mouse following intravenous immunisation with a low dose of SRBC is a good model of T-lymphocyte-mediated recruitment of myelomonocytic cells (21-22). The main features of this DTH are a maximal

specific response 4 d after active immunisation, which reaches a peak at approximately 20 hrs after ag challenge. This SRBC-specific response can be transferred systemically or locally to naive, syngeneic mice only by transfer of cell suspensions containing Thy 1⁺, CD 4⁺ lymphocytes from SRBC-sensitised mice (22). The adoptive DTH response is not detectable when sensitised T-lymphocytes are transferred to mice that have been lethally irradiated 30 hrs before transfer. In such recipients in which the bone marrow and blood myelomonocytic cell pool is depleted, the DTH response is restored only by the i.v. injection of bone marrow cells (22). We have therefore examined the importance of CR3-mediated myelomonocytic adhesion in this immunologically specific inflammatory response.

Figure 4 shows the increase in footpad thickness seen after local ag challenge with SRBC 4 d after active systemic immunisation. Naive animals (bar A) showed a negligible increase in footpad thickness of 0.10 ± 0.05 mm 20 hrs after ag challenge compared with the significant increase of 1.26 ± 0.28 mm seen in the footpad size of the actively immunised control mice (Bar B). The degree of increased footpad thickness was dependent upon the amount of ag injected as the high dose of SRBC (10^8 cells/footpad, Bar B) elicited 2.3X the swelling seen with a 10-fold lower amount of ag (0.54 ± 0.16 mm, Bar F). Intravenous injection of 1mg of 5C6 mAb at the time of ag challenge completely abolished the footpad swelling seen at 20 hrs in both high (Bar C) and low dose (Bar G) ag challenges. Delay of the i.v. injection of 5C6 mAb for up to 6 hrs after ag challenge still led to highly efficient inhibition of the footpad swelling at 20 hrs (Bar D). The specificity of the 5C6 IgG effect is shown in Table IV where the inhibitory effect of 5C6 IgG on footpad swelling is compared with buffer alone, a control rat mAb 7/4 binding to circulating myelomonocytic cells with a similar size number to 5C6 (21) or the pepsin F(ab')₂ fragment of 5C6, none of which inhibited footpad swelling. The half-life of injected 5C6 IgG and F(ab')₂ was found to 7.5h and < 2hrs respectively.

TABLE IV

Increase in footpad thickness 20 hours after antigenic challenge
with SRBC

<u>Immunisation</u> (day 0)	<u>Challenge</u> (day 4)	<u>Ab i.v.</u>	<u>Increase in Footpad Thickness</u>
(i.v.)	(sc)		(mm)
PBS	SRBC	-	0.05 ± 0.10
SRBC	SRBC	-	1.05 ± 0.10
SRBC	SRBC	5C6 IgG	0.10 ± 0.05
SRBC	SRBC	5C6 F(ab') ₂	0.90 ± 0.20
SRBC	SRBC	7/4 IgG	1.05 ± 0.17

Assay as described in Materials and Methods. Antibodies (1mg) were injected intravenously at the time of ag challenge. Results show the mean ± SD of 4 mice in each group from one representative experiment.

The ability of 5C6 to inhibit the DTH at 20 hrs was confirmed on histological examination of the footpad sections. The 20 hrs response to injected SRBC in a naive mouse shows intact SRBC in the extravascular tissue spaces. The host response to these erythrocytes was minimal with an occasional infiltrating neutrophil or monocyte. In contrast, the actively sensitised mouse challenged with SRBC in the absence of 5C6 mAb shows an obvious inflammatory infiltrate at 20 hrs, comprised of a mixture of monocytes and neutrophils. In actively immunised mice injected i.v. with 5C6 IgG at the time or up to 6 hrs after ag challenge, intact SRBC are seen dispersed in the extravascular tissue space without any accompanying myelomonocytic inflammatory infiltrate.

The CR3-dependent and CR3-independent recruitment of myelomonocytic cells to DTH

Fig. 5 illustrates the time course of the footpad swelling seen after ag challenge of actively immunised mice in the presence or absence of 5C6. The control sensitised mice showed significant increases in footpad thickness within 12 hrs of ag challenge. This swelling then reached a peak at 24 hrs, subsided to 42% of the peak level at 48 hrs and returned to the baseline at 72 hrs. Mice injected with 5C6 at the time of ag challenge had an increase in footpad size of 0.08 ± 0.07 mm at 24 hrs compared to 0.90 ± 0.17 mm of the sensitised controls. By 48 hrs, the 5C6-treated mice had an increase in footpad size of 0.39 ± 0.07 mm which was not different to the declining increase of 0.38 ± 0.05 in the untreated sensitised mice. From 48 hrs onwards, the curves of the 5C6-treated and the control mice were virtually superimposable. A second injection of 5C6 20hrs after ag challenge failed to alter appreciably the late increase in footpad size seen at 48 hrs in the 5C6 treated mice. Naive animals injected with SRBC in the footpad showed no increase in footpad size at any time.

Circulating levels of 5C6 IgG following a single injection of 1mg IgG were analysed by serial dilution of plasma samples. The anti-adhesive titre of the mAb was assayed in vitro using either bone marrow neutrophils or elicited M ϕ as described in Example I

and functionally saturating blood levels were maintained for at least 72 hrs. FACS analysis of 5C6 binding to blood, bone marrow and peritoneal cavity cells after a single i.v. injection of 5C6 showed that saturation binding to blood myelomonocytic cells was achieved within 1 hr of injection and maintained for 72 hrs. At no stage was any evidence found of leukocyte depletion, cytotoxicity or alterations in the differential blood count. Bone marrow labelling was weak at 1 hr, reached saturation by 24 hrs and then remained stable for the duration of the experiment. We therefore conclude that adequate levels of circulating 5C6 IgG were maintained in terms of both functional and binding criteria for the duration of the experiment and that inadequate concentrations of mAb do not account for the failure of 5C6 to inhibit the DTH at 48 hrs.

The effect of 5C6 during the time course is once again easily visible on histological examination. At 20 hrs the inhibition of inflammatory recruitment by 5C6 was complete. A control footpad from an actively sensitised mouse examined at 48 hrs shows a florid inflammatory myelomonocytic infiltrate. There are very few intact SRBC visible in the tissue spaces and there are many highly phagocytic cells filled with erythrocyte debris. In contrast, the 5C6-injected mice showed a small inflammatory infiltrate at 48 hrs sufficient to account for the swelling seen at that time. Most of the ag remained intact in the extravascular tissue spaces.

We conclude that the recruitment of myelomonocytic cells to the challenged footpad is biphasic in actively immunised animals. It consists of an early CR3-dependent phase that accounts for most of the footpad swelling and the bulk of inflammatory cell recruitment. There is a second CR3-independent phase that accounts for the modest swelling and cellular infiltration seen at 48 hrs, the time at which the increased footpad thickness in 5C6-treated and control mice becomes indistinguishable, although obvious differences in both cellular infiltration and ag persistence remain.

Local and systemic administration of 5C6 in naive, syngeneic mice.Effects on local transfer of DTH

Although CR3 is restricted to the myelomonocytic lineage and is not present on resting or activated lymphocytes, it is important to demonstrate that the 5C6 mAb limits its effects to inhibition of myelomonocytic recruitment and does not impair the ability of T-lymphocytes to become activated and transfer DTH. To assess the effects of 5C6 on the development of functional sensitised T-cells, mice were injected i.v. with 5C6 and then immunised with SRBC. Four days later, sensitised T-cells were harvested, washed by centrifugation, mixed with SRBC and injected into the footpad of naive, syngeneic mice. Table V shows that the footpad swelling seen after passive transfer of ag and blood leukocytes was similar using donor leukocytes recovered from mice immunised with SRBC in the presence or absence of systemic 5C6.

TABLE V

mAb 5C6 inhibits T-cell-dependent myelomonocytic recruitment but not
ag-dependent T-cell priming or activation

<u>Treatment (donors)</u>	<u>Transfer to recipients</u>	<u>Increase footpad thickness (recipients) at 15 hrs (mm)</u>
PBS	Blood leukocytes)
"	SRBC) 0.10 ± 0.05
"	Blood leukocytes + SRBC)
SRBC	Blood leukocytes) 0.10 ± 0.05
"	SRBC)
"	Blood leukocytes + SRBC	0.52 ± 0.05
"	Blood leukocytes + SRBC Recipients treated 5C6 IgG i.v.	0.10 ± 0.08
"	Blood leukocytes + SRBC Transferred cells pre- incubated 5C6 and washed before transfer	0.58 ± 0.09
SRBC + 5C6 IgG	Blood leukocytes) 0.08 ± 0.06
" "	SRBC)
" "	Blood leukocytes + SRBC	0.65 ± 0.12

Performed as described in Materials and Methods. Results reflect the mean ± SD of 4 mice per group. Equivalent results were obtained in each of 2 separate experiments.

We also examined the effect of 5C6 injection in recipient mice on hypersensitivity following passive local transfer of SRBC-reactive T-cells and SRBC. Local transfer led to acute onset of DTH and reached a peak at 15 hrs after ag challenge.

Table II shows that local transfer to naive, syngeneic recipients of sensitised blood leukocytes mixed with SRBC led to an increase in footpad size of 0.52 ± 0.05 mm in control mice at 15 hrs whilst leukocytes or ag alone failed to elicit any response. If mice were injected with 5C6 i.v. at the time of local transfer of sensitised cells and ag, increase in footpad size was significantly diminished at 0.10 ± 0.08 mm at 15 hrs. This result was confirmed by histologic observation.

Effect of 5C6 on adoptive transfer of T-cell-dependent inflammatory reaction to tuberculin

The effect of 5C6 was not restricted to inhibiting T-cell-dependent recruitment of inflammatory cells to one ag alone at a single anatomical site. We have analysed recruitment of monocytes to the peritoneal cavity following simultaneous injection of BCG-sensitised T-lymphocytes and tuberculin. This system has the advantage of allowing sampling of peritoneal cells and accurate assessment of the number of inflammatory cells recovered. We chose to examine the cellular response at 2 d to highlight the CR3-dependent recruitment of monocytes.

Fig. 6 shows the effects of 5C6 on the inflammatory recruitment following transfer of sensitised lymphocytes and tuberculin to the peritoneal cavity of naive, syngeneic mice. Naive mice not injected with cells or ag, or injected with either cells or ag alone, yielded $4.65 \pm 1.68 \times 10^6$ peritoneal leukocytes of which 45% (2.1×10^6) were M ϕ by virtue of their expression of the F4/80 ag by immunocytochemistry. 2% were neutrophils and 53% lymphocytes. 48 hrs after transfer of 1.5×10^7 BCG-sensitised lymph node cells and 50 μ g tuberculin, the number of peritoneal cells had increased to $9.12 \pm 2.29 \times 10^6$ cells, of which 66% (6.10×10^6) were M ϕ , 8% neutrophils and 26%

lymphocytes. In contrast, mice treated by the i.v. injection of 5C6 at the time of transfer of sensitised cells and tuberculin had only $4.28 \pm 2.22 \times 10^6$ peritoneal leukocytes at 48 hrs of which 47%: (2.01×10^6) were M ϕ , 3% neutrophils and 50% lymphocytes. Injection of tuberculin alone failed to elicit any quantitative or qualitative changes in the recovered cell population. The cellular response to the local injection of BCG-sensitised T-cells mixed with tuberculin was predominantly monocytic in nature and was completely inhibited by the i.v. injection of 5C6 mAb, if the initial resident population is taken into account.

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CLAIMS

1. An anti-CR3 specific antibody for use in the inhibition of recruitment of myelomonocytic cells to inflammatory stimuli.
2. An antibody according to Claim 1 which is capable of inhibiting the attachment of myelomonocytic cells to bacteriologic plastic.
3. An anti-CR3 specific antibody characterised in that the antibody is capable of inhibiting the attachment of myelomonocytic cells to bacteriologic plastic.
4. An antibody according to Claim 2 or 3, which inhibits the adhesion of myelomonocytic cells to bacteriologic plastic to the extent of at least 85%.
5. An antibody according to any of the preceding claims which causes detachment of myelomonocytic cells from bacteriologic plastic.
6. An antibody according to Claim 5, which is capable of detaching at least 50% of thioglycollate-elicited peritoneal macrophages from bacteriologic plastic.
7. An antibody according to any of the preceding claims which has specificity for the α subunit of CR3.
8. A method for the preparation of a cell line which produces an antibody according to Claim 3 comprising preparing a plurality of cell lines which produce anti-CR3 specific antibodies, screening the cell lines for antibodies which inhibit adhesion of myelomonocytic cells to bacteriologic plastic and selecting a cell line which produces an antibody which inhibits adhesion.
9. A cell line when prepared by a method according to Claim 8.

10. A therapeutic composition comprising an antibody according to Claim 1 or Claim 3 in conjunction with a suitable excipient diluent or carrier.
11. A therapeutic composition according to Claim 10 for parenteral administration.
12. A method for the preparation of a therapeutic composition according to Claim 10, comprising admixing an antibody according to Claim 1 or 3 with a suitable excipient, diluent or carrier.
13. The use of an anti-CR3 specific antibody in the preparation of a medicament for inhibiting the recruitment of myelomonocytic cells to inflammatory stimuli.
14. A method of therapy for inhibiting recruitment of myelomonocytic cells to inflammatory stimuli in a human or animal subject comprising administering to the subject an effective amount of an anti-CR3 specific antibody.
15. A method according to Claim 14 for the treatment or prophylaxis of a disease which involves recruitment of myelomonocytic cells in delayed type hypersensitivity reactions mediated by T-lymphocytes.
16. A method according to Claim 15 for treatment or prophylaxis of chronic inflammation, drug induced hypersensitivity reactions, rheumatoid arthritis, immune vasculity, glomerulonephritis or inflammatory bowel disease.
17. The use of an anti-CR3 specific antibody in the treatment or prophylaxis of a disease or disease state which arises as the result of the recruitment of myelomonocytic cells to inflammatory stimuli.

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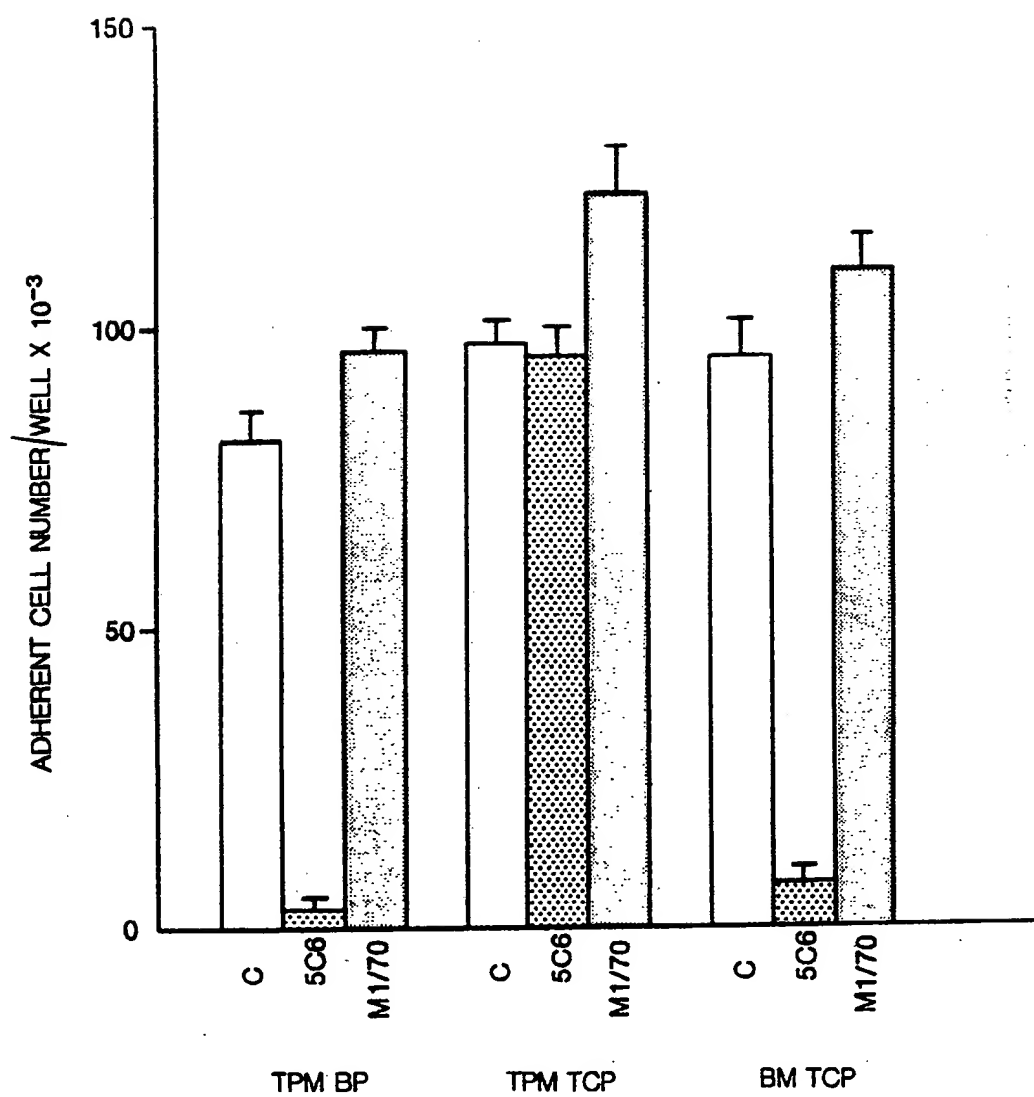


FIG. 1

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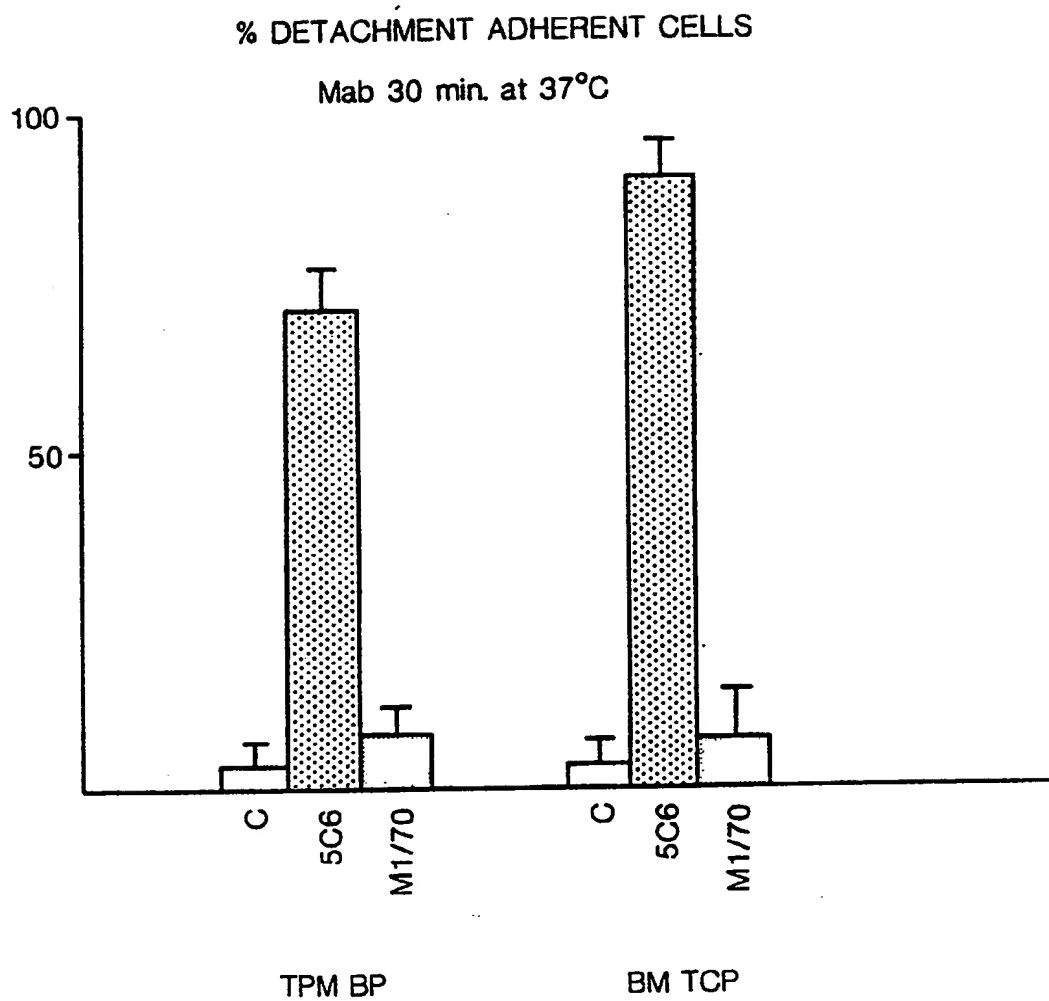


FIG. 2

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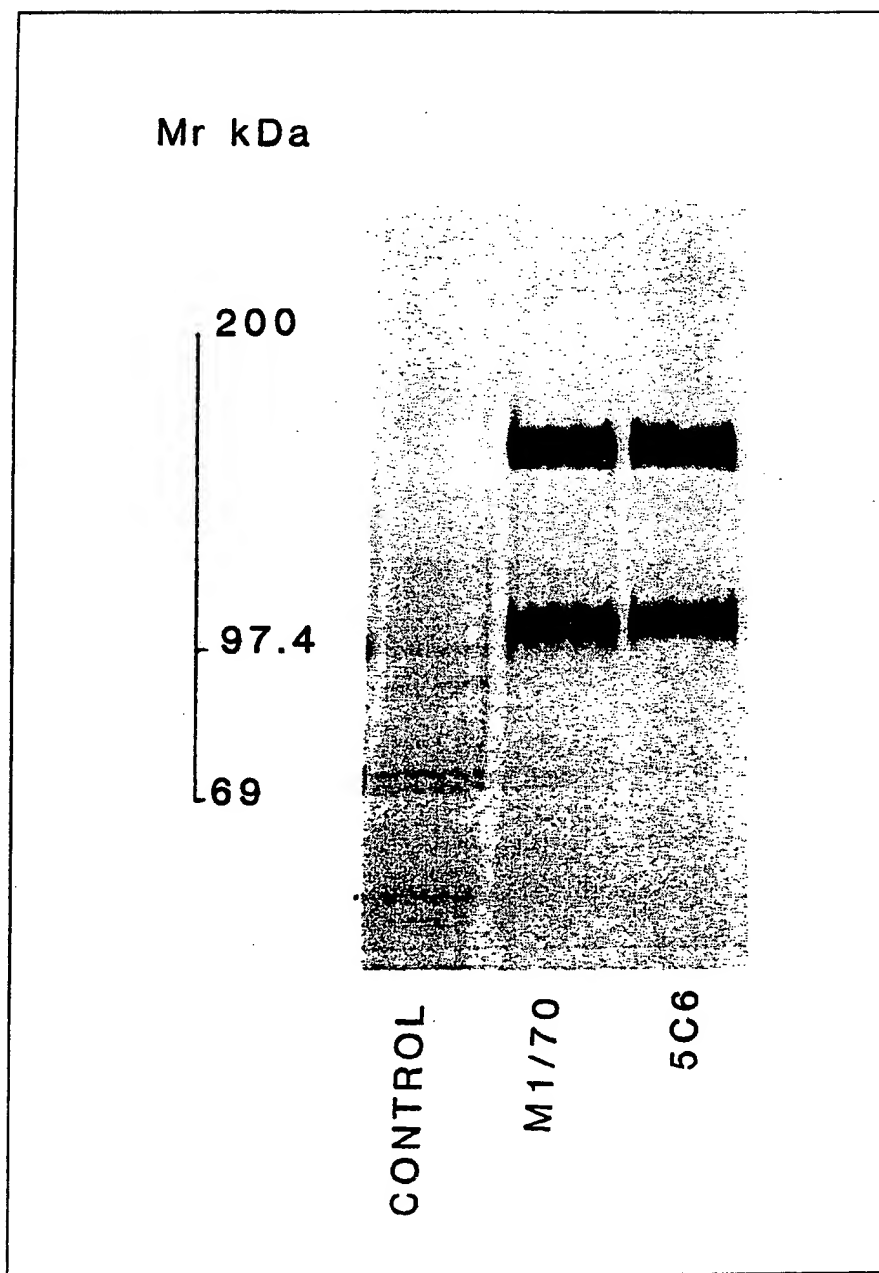


FIG. 3

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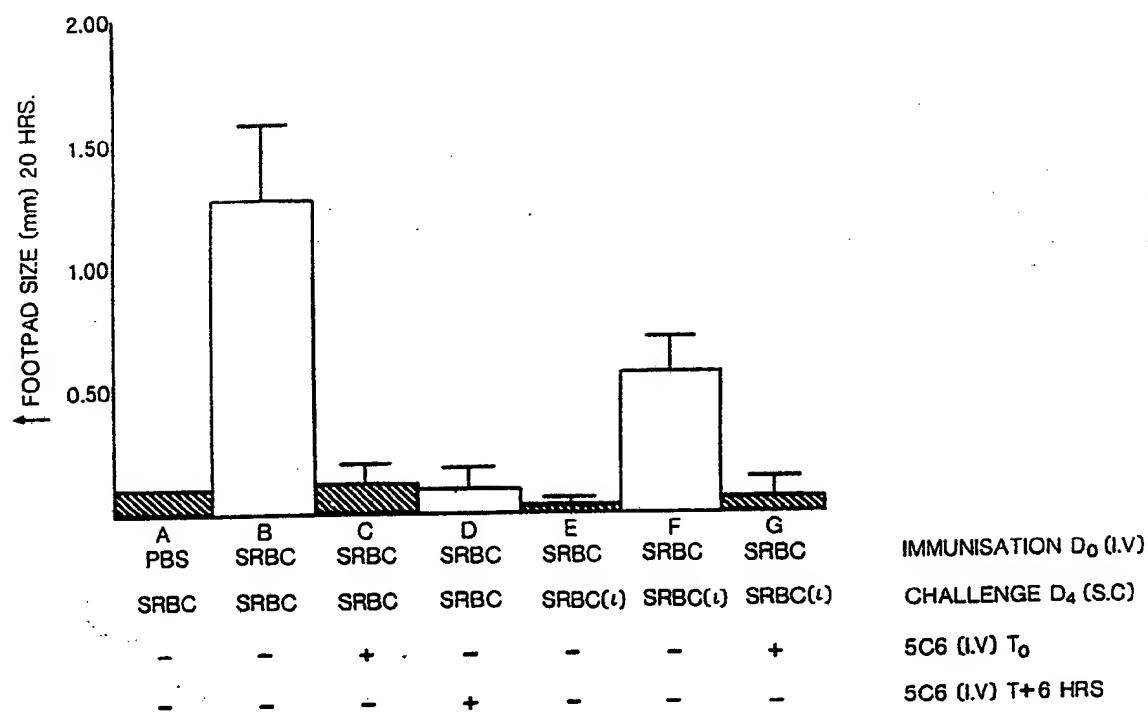


FIG. 4

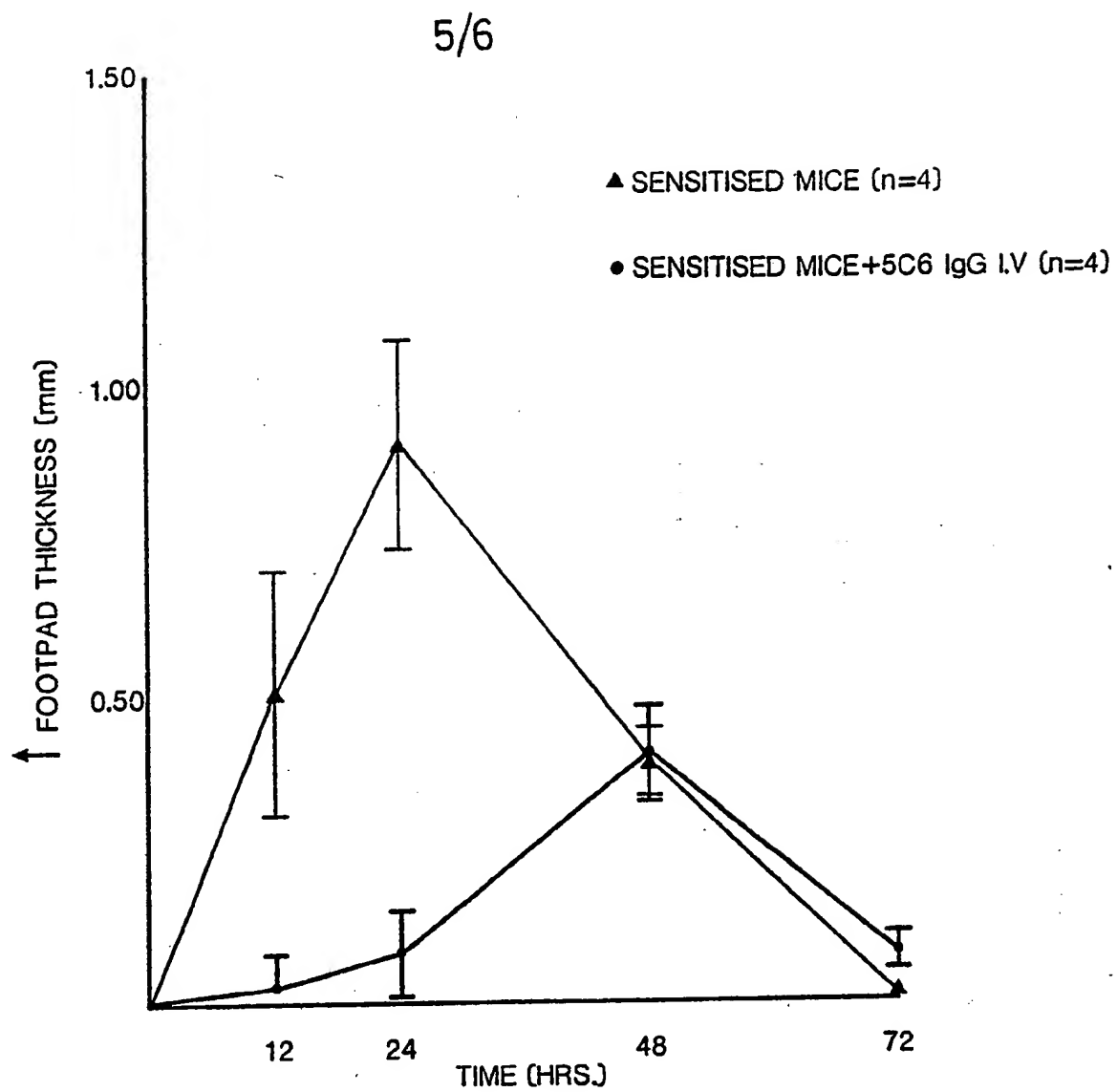


FIG. 5

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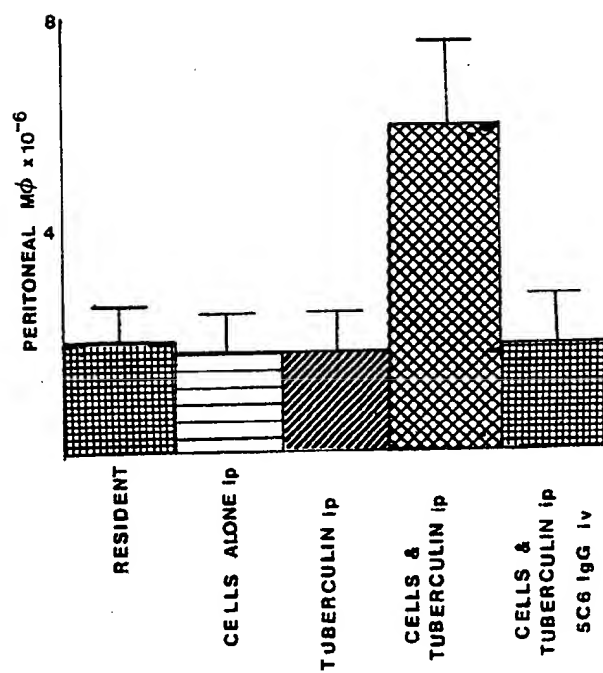


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB88/00977

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : A 61 K 39/00, C 12 P 21/00, C 07 K 15/06, C 12 N 5/00, C 12 N 15/00														
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC⁴</td> <td style="padding: 5px;">A 61 K</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁴	A 61 K								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 15%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X Y</td> <td style="padding: 5px;"> Dialog Information Services, File 154: Medline 83-89, Dialog accession no. 06361281, Pham Huu Tet al: "Comparison of blocking effects of monoclonal antibodies anti MO1-alpha and anti-LFA1-alpha on human neutrophil functions", & Immunology Sep 1987, 62 (1) p. 61-7 see the whole document -- </td> <td style="vertical-align: top; padding: 5px;"> 1-7, 10-13 8, 9 </td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">X Y</td> <td style="padding: 5px;"> Dialog Information Services, File 154: Dialog accession no. 05924525, Anderson D.C. et al.: "Contributions of the Mac-1 glyco- protein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit-specific mono- clonal antibodies", & J Immunol Jul 1 1986, 137 (1) p. 15-27 see the whole document -- </td> <td style="vertical-align: top; padding: 5px;"> 1, 7 2-6, 8-13 </td> </tr> <tr> <td colspan="3" style="text-align: center; padding: 5px;">-/-</td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X Y	Dialog Information Services, File 154: Medline 83-89, Dialog accession no. 06361281, Pham Huu Tet al: "Comparison of blocking effects of monoclonal antibodies anti MO1-alpha and anti-LFA1-alpha on human neutrophil functions", & Immunology Sep 1987, 62 (1) p. 61-7 see the whole document --	1-7, 10-13 8, 9	X Y	Dialog Information Services, File 154: Dialog accession no. 05924525, Anderson D.C. et al.: "Contributions of the Mac-1 glyco- protein family to adherence-dependent granulocyte functions: structure-function assessments employing subunit-specific mono- clonal antibodies", & J Immunol Jul 1 1986, 137 (1) p. 15-27 see the whole document --	1, 7 2-6, 8-13	-/-		
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the International filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; margin-top: 10px;">31st January 1989</div> </td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; margin-top: 10px;">27 FEB 1989</div> </td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; margin-top: 10px;">EUROPEAN PATENT OFFICE</div> </td> <td style="border-bottom: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> P.C.G. VAN DER PUTTEN </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; margin-top: 10px;">31st January 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; margin-top: 10px;">27 FEB 1989</div>	International Searching Authority <div style="text-align: center; margin-top: 10px;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> P.C.G. VAN DER PUTTEN </div>								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<p>Dialog Information Services, File 154: Dialog accession no. 06359731, Keizer G.D. et al.: "Role of p150,95 in adhesion, migration, chemotaxis and phagocytosis of human monocytes", & Eur J Immunol Sep 1987, 17 (9) p. 1317-22 see the whole document</p> <p>--</p>	1-9.
X Y	<p>Blood, Vol. 67, No. 4, April 1986, pages 1007-1013, W.J. Wallis et al.: "Monoclonal Antibody- -Defined Functional Epitopes on the Adhesion- Promoting Glycoprotein Complex (CDw18) of Human Neutrophils", see the whole document, see in particular page 1007 and "Discussion"</p> <p>--</p>	1, 7, 10-13 2-6, 8, 9
X Y	<p>Blood, Vol. 69, No. 4, April 1987, pages 1167-1174, G. ISmail et al.: "Prevention of Pulmonary Injury in Isolated Perfused Rat Lungs by Activated Human Neutrophils Preincubated With Anti-Mol Monoclonal Antibody", see the whole document</p> <p>--</p>	1, 7 2-6, 8-13
X Y	<p>The Journal of Immunology, Vol. 136, No. 12, 15 June 1986, pages 4548-4553, T.H. Pohlman et al.: "An Endothelial Cell Surface Factor(S) Induced in Vitro by Lipo- polysaccharide, Interleukin 1, and Tumor Necrosis Factor-α Increases Neutrophil Adherence by a CDw18-Dependent Mechanism", see the whole document, in particular page 4552</p> <p>--</p>	1, 7 1-6, 8-13
A	<p>Blood, Vol. 69, No. 1, January 1987, pages 338-340, K-E Arfors et al.: "A Monoclonal Antibody to the Membrane Glycoprotein Complex CD18 Inhibits Polymorphonuclear Leukocyte Accumulation and Plasma Leakage In Vivo", see page 340</p> <p>--</p>	1-13
A	<p>Nature, Vol. 314, 11 April 1985, pages 540-542, T.A. Springer et al.: "Sequence homology of the LFA-1 and Mac-1 leukocyte adhesion glyco- proteins and unexpected relation to leukocyte interferon", see the whole document</p> <p>--</p>	1-13

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,P	<p>Dialog Information Services, File 154: Dialog accession no. 06416080, H. Rosen et al.: "Monoclonal antibody to the murine type 3 complement receptor inhibits adhesion of myelomonocytic cells in vitro and inflammatory cell recruitment in vivo", & J Exp Med (UNITED STATES) Dec 1 1987, 166 (6), p. 1685-701 see the whole document</p> <p style="text-align: center;">--</p>	1-13
A,P	<p>WD, A1, 8806592 (DANA FARBER CANCER INSTITUTE) 7 September 1988 see the whole document</p> <p style="text-align: center;">-----</p>	1-7

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